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MALIC ENZYME AND AMINOTRANSFERASES IN RELATION TO 3-PHOSPHOGLYCERATE FORMATION IN PLANTS WITH THE C_4-DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS

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Abstract—In plants with the C_4-dicarboxylic acid pathway an inverse relationship was found between the content of malic enzyme in leaves and that of aspartate and alanine aminotransferases. Some species contained adequate malic enzyme to support the decarboxylation of malate in the bundle sheath chloroplasts as part of the major route of carbon flow. Other species with low malic enzyme activity contained 5–10 times more aspartate aminotransferase and about 50 times more alanine aminotransferase. These aminotransferases were about equally distributed between the mesophyll and the bundle sheath cells. We propose that in the latter species aspartate rather than malate is transported to the bundle sheath chloroplasts. The results are most compatible with aspartate being converted to oxaloacetate in the bundle sheath chloroplasts and the latter compound being decarboxylated to provide CO_2 for 3-phosphoglycerate formation.

INTRODUCTION

IN PLANTS with the C_4-dicarboxylic acid pathway of photosynthesis[1,2] essentially all of the externally-derived CO_2 incorporated into the C-1 of 3-phosphoglycerate (3-PGA) enters via the C-4 carboxyl of C_4-dicarboxylic acids.[3–6] We have considered two possible mechanisms for the transfer of the C-4 of dicarboxylic acids. One implicated a transcarboxylation reaction and the other the decarboxylation of a dicarboxylic acid and the fixation of the released CO_2 by ribulosediphosphate(RuDP)carboxylase.[1,2] Previously, the former mechanism was favoured but only on the basis of negative evidence. This included evidence against the existence of an intermediate pool of free CO_2 within the leaf[3,4] and evidence that the activity of RuDP carboxylase was apparently too low to have a major quantitative

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[1] M. D. HATCH and C. R. SLACK, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, p. 35, Interscience, London (1970).
[2] M. D. HATCH and C. R. SLACK, *Ann. Rev. Plant Physiol.* **21**, 141 (1970).
[3] M. D. HATCH and C. R. SLACK, *Biochem. J.* **101**, 103 (1966).
[4] H. S. JOHNSON and M. D. HATCH, *Biochem. J.* **114**, 127 (1969).
[5] M. D. HATCH, C. R. SLACK and T. A. BULL, *Phytochem.* **8**, 697 (1969).
[6] C. B. OSMOND and P. N. AVADHANI, *Plant Physiol.* **45**, 228 (1970).

role in photosynthesis.[7,8] However, it has now been demonstrated that species with the C_4-dicarboxylic acid pathway contain RuDP carboxylase activities comparable to the photosynthesis rates for these plants.[9,10] This enzyme is located in the bundle sheath chloroplasts whereas the enzymes implicated in the fixation of CO_2 into oxaloacetate are restricted to the mesophyll chloroplasts.[11,12] These findings would be consistent with RuDP carboxylase having a major role in the C_4-dicarboxylic acid pathway and with the operation of a process involving decarboxylation of a C_4-dicarboxylic acid followed by refixation of the released CO_2 into 3-PGA. The present paper describes studies on the activity and location of enzymes involved in the formation, interconversion and decarboxylation of C_4-dicarboxylic acids. The evidence suggests the operation of two separate routes for transport and decarboxylation of C_4-dicarboxylic acids. Most plant species can be clearly divided according to which of these processes is dominant.

RESULTS

Comparative Activities of Malic Enzyme and Aminotransferases

We have previously reported that the malic enzyme activity in some grasses with the C_4-dicarboxylic acid pathway is about 50 times that for Calvin cycle plants.[7] The fact that this enzyme is located in the bundle sheath chloroplasts, together with RuDP carboxylase, led to the suggestion that malate could act as a carrier of CO_2 between the mesophyll and bundle sheath chloroplasts.[12,13] However, as briefly reported elsewhere, some other

TABLE 1. MALIC ENZYME ACTIVITY IN THE LEAVES OF C_4-DICARBOXYLIC ACID PATHWAY PLANTS AND ITS RELATION TO OTHER ENZYME ACTIVITIES

Group	Plant species	Enzyme activity* (μmole/min/mg chlorophyll)				
		Malic enzyme	NADP-malate dehydrogenase	Alanine aminotransferase	Aspartate aminotransferase	RuDP carboxylase
1	*Zea mays*	10	9	0·7	5·7	2·5
	Saccharum (hybrid)	12	12	0·3	4·6	2·3
	Sorghum (hybrid)	10	14	0·6	4·9	2 6
2	*Gomphrena celosoides*	5·5	2·3	16	15	—
3	*Eragrostis brownii*	0 4	0·6	16	—	2·4
	Chloris gayana	0 2	4·0	10	22	2·3
	Amaranthus edulis	0·8	1·5	21	28	4 4
	Amaranthus palmeri	1·3	2·4	32	38	3·2
	Atriplex nummularia	0·09	2·8	26	41	0·5
	Atriplex spongiosa	0·15	2·1	25	40	2·8

* Procedures for extracting and assaying enzymes are described in the Experimental. RuDP carboxylase was assayed at 30° and the remaining enzymes at 22°. For comparison the maximum photosynthesis rates for several C_4-dicarboxylic acid pathway plants range between 3 and 5 μmoles CO_2/min/mg chlorophyll (see Refs. 7 and 8).

† With this species the bundle sheath cells were probably not completely extracted (see Ref. 10).

[7] C. R. SLACK and M. D. HATCH, *Biochem. J.* **103,** 660 (1967).
[8] H. S. JOHNSON and M. D. HATCH, *Phytochem.* **7,** 375 (1968).
[9] O. BJORKMAN and E. GAUHL, *Planta* **88,** 197 (1969).
[10] T. J. ANDREWS and M. D. HATCH, *Phytochem.* **10,** 9 (1971).
[11] C. R. SLACK, *Phytochem.* **8,** 1387 (1969)
[12] C. R. SLACK, M. D. HATCH and D. J. GOODCHILD, *Biochem. J.* **114,** 489 (1969).
[13] J. A. BERRY, W. J. S. DOWNTON and E. B. TREGUNNA, *Can. J. Botany* **48,** 777 (1970).

C_4-dicarboxylic acid pathway species contain only low activities of malic enzyme.[1,14] In Table 1 the activity of malic enzyme in several species with the C_4-dicarboxylic acid pathway are compared with those for RuDP carboxylase and other enzymes implicated in the metabolism of C_4-dicarboxylic acids. Grasses of the Panicoid subfamily (Group 1) contained malic enzyme activities well in excess of observed photosynthesis rates. However, the activity in the species of Group 3 was only between 1% and 10% of that for the Group 1 species while an intermediate value was obtained for *Gomphrena celosoides*. There was a correlation between the activities of malic enzyme and NADP-malate dehydrogenase.

If malate is transported to the bundle sheath chloroplasts in species with high malic enzyme activity then alternative processes may operate in the species of Group 3. The possibility that aspartate may replace malate was tested by comparing the activity of aminotransferases in the different species. We showed earlier that the species of Group 1 have more than twice the aspartate aminotransferase activity of Calvin cycle plants.[7] However, the activity of this enzyme in Group 3 species was in turn many-fold higher than for Group 1 and even greater differences were observed for alanine aminotransferase. These activities were recorded with 2-oxoglutarate as the amino group acceptor. It should be noted that the aspartate:pyruvate aminotransferase activity in extracts of *Amaranthus palmeri* and *Atriplex spongiosa* was only between 1% and 2% of the activities recorded for asparate and alanine with 2-oxoglutarate as the acceptor.

The simplest hypothesis consistent with these results would be that species with low malic enzyme activity fix carbon into aspartate which is then transported to the bundle sheath chloroplasts and decarboxylated. The other product alanine would presumably be transported back to the mesophyll chloroplasts and there converted to pyruvate by alanine aminotransferase. On this basis attempts were made to locate aspartate decarboxylase activity in *Amaranthus* and *Atriplex* leaf extracts. No activity was detected.

Inter- and Intracellular Location of Aminotransferases and Other Enzymes

Density fractionation of leaf extracts in non-aqueous media has already been used to determine the location of several key enzymes of the C_4-dicarboxylic acid pathway.[11,12] This technique was used to provide information about the location of the enzymes being studied during the present investigation. As explained in detail elsewhere,[12] the leaves used were pretreated to ensure that either they were starch-free or starch had accumulated in the bundle sheath chloroplasts. In the former case both mesophyll and bundle sheath chloroplasts appear in lower density fractions while in the latter case the bundle sheath chloroplasts appear in fractions of higher density.

With destarched maize leaves, the distribution of aspartate aminotransferase paralleled that of chlorophyll and enzymes previously shown to be associated with chloroplasts (Table 2). However, little if any of the alanine aminotransferase was associated with chloroplasts. In view of the relatively low content of this enzyme in maize it would be reasonable to assume that much of this activity is operational at sites not directly connected with photosynthesis. The results for starch-containing maize leaves indicate that aspartate aminotransferase is associated with the mesophyll chloroplasts, its distribution being very similar to that of NADP-malate dehydrogenase which is located in the mesophyll chloroplasts in maize.[12]

A species representative of those with high aminotransferase activity (Group 3, Table 1),

[14] H. S. JOHNSON and M. D. HATCH, *Biochem. J.* **119**, 273 (1970).

TABLE 2. DISTRIBUTION OF CHLOROPHYLL AND ENZYMES IN FRACTIONS OF DESTARCHED AND STARCH-CONTAINING MAIZE LEAVES PREPARED IN NON-AQUEOUS MEDIA

	% of total in fractions of different density				
Enzyme*	< 1·30	1·30 −1·33	1·33 −1 36	1·36 −1·40	> 1·40
Chlorophyll	48(44)	13(17)	13(4)	17(22)	7(13)
NADP-malate dehydrogenase	50(47)	14(23)	9(7)	20(12)	6(11)
Malic enzyme	54(2)	16(6)	12(7)	12(57)	6(28)
Aspartate aminotransferase	51(47)	16(22)	12(9)	14(10)	7(12)
Alanine aminotransferase	16(7)	7(7)	13(5)	38(31)	26(50)

* Previous studies (see Refs. 11 and 12) with maize have shown that NADP-malate dehydrogenase is located in the mesophyll chloroplasts while malic enzyme, is exclusive to the bundle sheath chloroplasts. The data for these enzymes is included for comparison.

† The values for starch-containing leaves are in brackets.

Amaranthus palmeri was also examined using the non-aqueous density fractionation procedure (Table 3). Over 70% of the chlorophyll and enzymes of Group 1, enzymes assumed to be located in chloroplasts,[11,12] appeared in fractions of density < 1·33. As observed earlier with maize[12] there was a fractional difference in behaviour of the mesophyll chloroplast enzyme pyruvate, P_i dikinase compared with the other enzymes. This was presumably due to small differences in the density of the two types of chloroplasts. In contrast to maize, however, the mesophyll chloroplasts are apparently slightly heavier in *Amaranthus*.

Acid phosphatase has been used previously[12] as an indicator of the behaviour of 'soluble' enzymes during non-aqueous density fractionation. The bulk of the activity of this type of enzyme invariably appears in fractions of highest density and this was also

TABLE 3. DISTRIBUTION OF CHLOROPHYLL AND ENZYMES IN FRACTIONS OF DESTARCHED *Amaranthus palmeri* LEAVES PREPARED IN NON-AQUEOUS MEDIA

		% of total in fractions of different density				
Group	Enzyme	< 1·30	1 30 −1 33	1·33 −1·36	1·36 −1 40	> 1·40
	Chlorophyll	52	23	8	5	12
1	RuDP carboxylase	56	21	4	4	15
1	Ru 5-P kinase	55	19	6	5	15
1	Pyruvate, P_i dikinase	39	28	11	9	13
2	PEP carboxylase	5	5	3	6	81
2	Acid phosphatase	3	3	3	5	86
3	NADP-malate dehydrogenase	26	29	11	8	26
3	Aspartate aminotransferase	24	22	11	11	31
3	Alanine aminotransferase	7	5	5	4	78

the case with *Amaranthus* (Table 3). Whether phosphopyruvate (PEP) carboxylase behaves in this way or appears associated with mesophyll chloroplasts depends on several factors.[12] We have suggested[12] that PEP carboxylase may be located on the chloroplast outer membrane, or associated reticulum, and that its appearance in the high-density fractions is related to the loss of this membrane from the organelles. With *Amaranthus* leaves little of this enzyme remained associated with chloroplasts (Table 3). A proportion of each of the enzymes in Group 3 of Table 3 was not associated with chloroplasts. From this data and that for Group 1 and Group 2 enzymes it was calculated that this proportion was about 20% for NADP-malate dehydrogenase, 33% for aspartate aminotransferase and 90% of the alanine aminotransferase. Of course, as already indicated, this component may have been originally associated with the outer membrane of chloroplasts. Similar results were obtained when the experiment described in Table 3 was repeated on another sample of *Amaranthus* leaves.

The non-aqueous density fractionation procedure can only provide information about the distribution of enzymes between mesophyll and bundle sheath cells if enzymes are associated with chloroplasts and remain so during isolation. To provide more information about the intercellular distribution of the aminotransferases we therefore employed the procedure described originally by Bjorkman and Gauhl.[9] With this technique a series of extracts are obtained by grinding leaves with increasingly vigorous procedures. The initial extract contains a proportionately greater part of the contents of the easily-broken mesophyll cells while the later extracts are enriched in bundle sheath contents.

The results of treating *Amaranthus* and *Atriplex* leaves in this way are shown in Table 4. Group 1 and Group 2 enzymes were included for comparison, being enzymes located exclusively in the bundle sheath and mesophyll cells respectively.[9,11,12] For the species examined we assumed that the proportion of the total activity of Group 3 enzymes (Table 4) not involved in photosynthesis would be quantitatively insignificant, and hence that essentially all the activity would be located in either the mesophyll or bundle sheath cells. This assumes, in turn, that the excess of activity of the aminotransferases in the species studied over that for Calvin cycle plants and other C_4-dicarboxylic acid pathway species (see Table 1) represents the component involved in photosynthesis. In the species examined this component would be equivalent to about 95% of the aspartate aminotransferase and 98% of the alanine aminotransferase activities.

Calculations from the data provided in Table 4 show that only about 65% of the NADP-malate dehydrogenase of *Amaranthus palmeri* leaves is associated with mesophyll cells. However, more than 90% of this enzyme was in the mesophyll cells of *Atriplex* leaves. The aminotransferases were about equally divided between the mesophyll and bundle sheath cells with both *Amaranthus* and *Atriplex*. Very similar results were obtained with *Amaranthus edulis*. In this species over 90% of the NADP-malate dehydrogenase and about 45% to 50% of the aminotransferases were located in the mesophyll cells.

For the studies described in Table 4 a sample of the homogenate obtained after each extraction was examined under the microscope. With both tissues the whole cells remaining after the second extraction consisted of small pieces of vascular tissue surrounded by rows of from three to eight intact bundle sheath cells. Few if any mesophyll cells were apparent. After mortar-grinding to obtain the third extract some fragments of vascular bundles were apparent but few intact bundle sheath cells remained associated with these fragments.

In the following section we suggest that in some species oxaloacetate is decarboxylated in the bundle sheath chloroplasts. However, to date we have not been able to clearly

TABLE 4. DISTRIBUTION OF ENZYMES IN EXTRACTS OBTAINED BY DIFFERENTIAL MACERATION OF *Amaranthus palmeri* AND *Atriplex spongiosa* LEAVES

Group	Enzyme	% of total in each fraction								Assumed (brackets) or calculated % in cells†			
		Amaranthus extracts*				*Atriplex* extracts*				*Amaranthus*		*Atriplex*	
		Total activity	1	2	3	Total activity	1	2	3	Meso-phyll	Bundle sheath	Meso-phyll	Bundle sheath
	Chlorophyll		25	55	20		52	28	20				
1	RuDP carboxylase	2·1	15	48	37	2·6	33	28	39	(0)	(100)	(0)	(100)
	Ru 5-P kinase	24	17	49	34	22	32	28	40	(0)	(100)	(0)	(100)
2	PEP carboxylase	4·9	35	58	7	5 6	98	2	0	(100)	(0)	(100)	(0)
	Pyruvate, P_i dikinase	1·1	32	59	9	0 8	97	3	0	(100)	(0)	(100)	(0)
3	NADP-malate dehydrogenase	2·4	28	55	17	2 2	89	8	3	65	35	91	9
	Alanine aminotransferase	32	25	50	25	28	63	18	19	44	56	47	53
	Aspartate aminotransferase	48	24	51	25	41	62	17	21	42	58	46	54

* Details of the preparation of extracts are provided in the Experimental. The total activity extracted is expressed as μmoles/min/mg chlorophyll.

† The assumed values are based on the results of previous studies (see text) The proportions of the Group 3 enzymes in the mesophyll and bundle sheath cells was calculated from the formula· $ax + b(1-x) =$ observed % of the enzyme in Extract 1.

Where: x is the proportion of the enzyme (as a decimal) in the mesophyll cells and hence $(1 - x)$ is the proportion in the bundle sheath cells.

a and b are proportions of the mesophyll and bundle sheath cells respectively broken during the preparation of Extract 1. These values are obtained from the percentages of Group 1 and Group 2 enzymes respectively in Extract 1.

Similar calculations were made using the proportions of enzymes in Extract 3. The separate determinations were in good agreement and the averages are quoted.

demonstrate an enzyme-catalysed decarboxylation of oxaloacetate in leaf extracts above the rapid nonenzymic decarboxylation that occurs at neutral pH in the presence of Mg^{2+} or Mn^{2+}.

DISCUSSION

RuDP carboxylase activity in C_4-dicarboxylic acid pathway species is located in the bundle sheath chloroplasts,[11,12] and is adequate to account for its integral operation in photosynthesis provided the enzyme is adequately provided with CO_2. Assuming that in these species 3-PGA is formed exclusively via RuDP carboxylase, then radiotracer studies have shown that CO_2 is supplied to this enzyme from the C-4 carboxyl of a C_4-dicarboxylic acid.[3–6] Presumably the advantage of such a process must be to provide a mechanism for concentrating CO_2 at the site of action of RuDP carboxylase. As discussed in detail elsewhere,[2] the extracted activity of RuDP carboxylase in all plants examined is quite inadequate to account for photosynthesis if the concentration of CO_2 *in vivo* is equal to or less than that in equilibrium with CO_2 in air. Therefore, in C_4-dicarboxylic acid pathway plants, there must be a mechanism for forming C_4-dicarboxylic acids in the mesophyll chloroplasts and transporting them to the bundle sheath chloroplasts. In addition, the bundle sheath chloroplasts must have an adequate capacity for decarboxylating one or more of these acids.

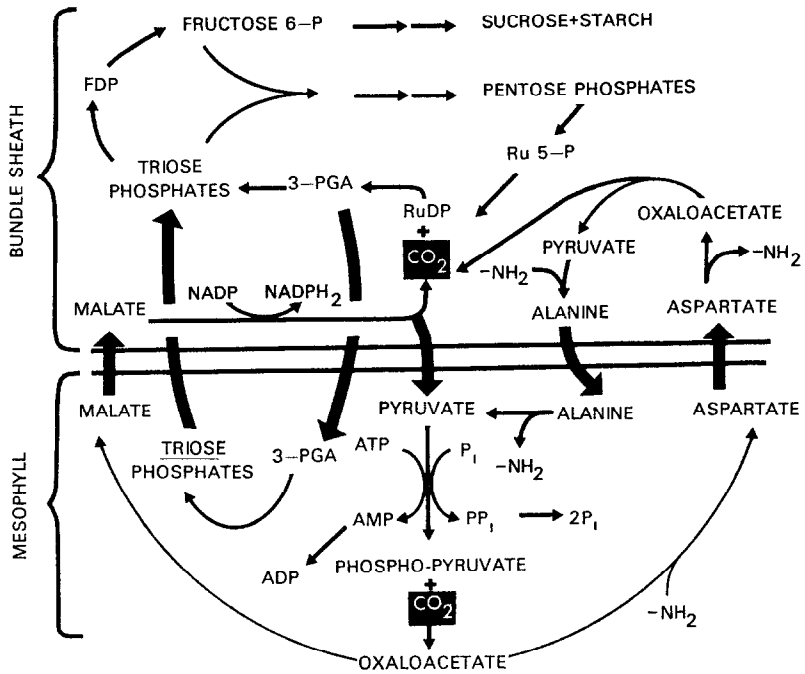

FIG. 1. PROBABLE REACTIONS AND INTERCELLULAR MOVEMENTS OF METABOLITES DURING THE OPERATION OF THE C_4-DICARBOXYLIC ACID PATHWAY.
The scheme, a modification of those presented previously shows reactions and intercellular movements of metabolites (heavy arrows) when either malate (left-hand side) or aspartate (right-hand side) is the C_4-dicarboxylic acid transported to the bundle sheath cells (see Refs. 1 and 12).

Our interpretation of the present data, together with that from previous studies is presented schematically in Fig. 1. This is a modification of those previously presented.[1,12] The Panicoid grasses (Group 1, Table 1) contain enzymes that fulfil the above requirements with respect to both activity and location. Thus, malate formed by NADP-malate dehydrogenase in the mesophyll chloroplasts could provide the CO_2 for 3-PGA formation by the combined action of malic enzyme and RuDP carboxylase in the bundle sheath chloroplasts. As we have previously indicated,[1,12] the operation of such a process would result in the transfer to the bundle sheath chloroplasts of reducing power as well as CO_2. The return of pyruvate to the mesophyll chloroplasts would be required to maintain carbon balance.

For each mole of malate decarboxylated in the bundle sheath chloroplasts 2 moles of 3-PGA but only 1 mole of $NADPH_2$ would be formed. Furthermore, the suggestion that the agranal bundle sheath chloroplasts of the Panicoid grasses may be unable to photoreduce NADP[12] is now supported by direct evidence for their lack of photosystem II and associated activities.[15,16] Apparently, therefore, only half of the 3-PGA formed could be reduced to triose phosphates in the bundle sheath chloroplasts. It is proposed that the remainder must be transported to and reduced in the mesophyll chloroplasts and then returned to the bundle sheath chloroplasts as triose phosphates (Fig. 1). The finding that

[15] W. J. S. DOWNTON, J. A. BERRY and E. B. TREGUNNA, *Z. Pflanzenphysiol.* **63**, 194 (1970).
[16] K. C. WOO, J. M. ANDERSON, N. K. BOARDMAN, W. J. S. DOWNTON, C. B. OSMOND and S. W. THORNE *Proc. Natl Acad. Sci. U.S.* **67**, 1825 (1970).

3-PGA kinase and NADP-glyceraldehyde phosphate dehydrogenase of maize are about equally distributed between the mesophyll and bundle sheath chloroplasts[12] is consistent with this view.

Other C_4-dicarboxylic acid pathway species contain only low malic enzyme activity (Group 3, Table 1) and possess granal bundle sheath chloroplasts[17] that are capable of photoreducing NADP.[16] The fact that these species contained much higher activities of aspartate and alanine aminotransferases suggested that they may transport CO_2 to the bundle sheath chloroplasts as aspartate rather than malate. However, if aspartate was then decarboxylated the aminotransferases would be required to operate only in the mesophyll cells; aspartate aminotransferase to form aspartate and alanine aminotransferase to regenerate pyruvate from alanine returned from the bundle sheath cells. However, we were unable to detect aspartate decarboxylase in leaf extracts and, of more significance, found the aminotransferases to be about equally distributed between the mesophyll and bundle sheath cells. This distribution of enzymes is more consistent with the view that aspartate must first be converted to oxaloacetate in the bundle sheath chloroplasts and that oxaloacetate is the acid decarboxylated. In order to maintain a balance of amino groups between the two types of cells alanine rather than pyruvate would have to be transported back to the mesophyll cells. Thus, alanine aminotransferase would also be required in both bundle sheath and mesophyll cells. Divalent metal ions catalyse a rapid decarboxylation of oxaloacetate and to date we have not been able to observe any enzyme-dependent activity above this rate with leaf extracts. Of course the reaction may be considerably more complex than we have assumed or a simple decarboxylation could be regulated by activators *in vivo*.

It is now clear that the anatomical and ultrastructural features which allow a division of species with the C_4-dicarboxylic acid pathway[1] are correlated with variations in the metabolic processes involved in CO_2 assimilation. These variations relate to whether malate or aspartate is transported from the mesophyll cells to the bundle sheath cells and are associated with marked differences in enzyme activities. In terms of enzyme activities most species fall fairly clearly into one or other of these two groups. However, *Gomphrena celosoides* provides one example where both processes may make substantial contributions.

EXPERIMENTAL

Plants were grown in soil or nutrient culture in a glasshouse except for the species of *Eragrostis* and *Chloris* which were obtained from the field. Mature leaves from relatively young plants were used.

Extraction of Enzymes from Fresh Tissue

For the exhaustive extraction of enzymes from leaves about 1·5 g of laminae were ground vigorously in a mortar for 90 sec at 0° with 4 vols (w/v) of 50 mM Tris HCl buffer, pH 8·3, 5 mM dithiothreitol and 2 mM EDTA and about 2 g of acid-washed sand. Small molecular weight materials were removed from these extracts by treating samples on small columns of Sephadex-G25 previously equilibrated with the above buffer solution.

For the stepwise extraction of tissue by differential maceration 2 g of leaves were blended initially for 10 sec at 0° with 15 ml of 50 mM Tris HCl buffer, pH 8·0, containing 5 mM dithiothreitol, 1 mM EDTA and 2 mM $MgCl_2$. The semi-micro cup of the Servall Omnimix was used with a blade speed about 40% of the maximum. The first extract comprised that material which passed through two layers of Miracloth The material retained was washed with an additional 10 ml of the above solution and was then transferred to the blender cup together with 15 ml of this solution. After blending at 0° for a further 40 sec at full speed the homogenate was again strained through Miracloth. After washing the material retained, as above, the final extract was obtained by grinding this material in a cooled mortar with sand Initially 3 ml of the above buffer mixture was added to give a thick slurry and after grinding for 90 sec an additional 3 ml was added followed by further grinding for 60 sec. A sample of each extract was treated on 6 ml column of Sephadex

[17] W. J. S. DOWNTON, *Can. J. Botany* (in press).

G-25, previously equilibrated with the above buffer mixture, to remove small molecular weight components present in the original extracts. Prior to taking samples for the spectrophotometric assay of enzymes the extracts were centrifuged at 10,000 *g* for 10 min.

Fractionation of Leaves in Non-aqueous Media

Leaves were frozen in liquid N_2 and then lyophilized, fractionated and stored as previously described.[12] The enzyme activity of each fraction was determined on solutions obtained by suspending the solid material in 0·1 M Tris HCl buffer, pH 8·0, containing 10 mM dithiothreitol and 2 mM EDTA. When pyruvate, P_i dikinase was to be determined 3 mM $MgCl_2$ was also included in the suspending solutions.

Assay of Enzymes

Except for the procedures described below the methods for assaying enzymes were as previously employed.[12]

For the assay of aspartate aminotransferase the decrease in absorbance of 340 mμ was measured in reactions containing, enzyme, 25 mM Tris HCl buffer, pH 8·0, 2·5 mM 2-oxoglutarate, 2·5 mM L-aspartate, 5 μg pyridoxal phosphate, 0·2 mM $NADH_2$, 2 mM EDTA and 5 units malate dehydrogenase in a total volume of 1 ml. The same system was used for alanine aminotransferase except that aspartate was replaced by L-alanine and malate dehydrogenase by lactate dehydrogenase.

Attempts to measure the decarboxylation of aspartate and oxaloacetate in leaf extracts were made by measuring the loss of radioactivity from ^{14}C-4-labelled substrates. Alternatively the spectrophotometric method of Kosicki[18] was used for measuring oxaloacetate decarboxylation.

The temperature was between 22° and 23° for spectrophotometric assays and 30° for other assays.

[18] G. W. Kosicki, *Biochem.* **7**, 4299 (1968).